ELISA Laboratory Protocols for the Plum Pox Virus National Surveillance Program.

Prepared by Laurene Levy, USDA-APHIS-PPQ, NPGQC, BARC-East, Bldg-580, Beltsville, MD 20705
Phone: 301-504-7157 Fax: 301-504-8539 Email: laurene.e.levy@usda.gov

Updated March 24, 2001

The following protocols are for the ELISA detection of plum pox virus in the *Prunus* samples collected during the year 2001 National Surveillance Program for Plum Pox Virus in the United States. The following are guidelines for sample collection, storage, processing, and testing.

A. Plum Pox Virus Testing General Information.

1. Plum pox virus (PPV) testing should begin when leaves are just fully expanded. The time when the first leaves are just fully expanded correlates to when PPV is reaching a high concentration in leaves. In some southern states that will be March, in most states that will be April or the beginning of May depending on *Prunus* development. Testing should begin in March or early April, and continue until June/July, or until consistent daily high temperatures of 32°C, or 89.6°F, are reached. With plum pox, as with most plant viruses, as high temperatures are reached the virus concentration decreases.

2. The kit we are using again for the year 2001 survey is the Durviz Plum Pox Virus ELISA kit which contains a monoclonal antibody that reacts with all plum pox virus isolates tested to date (see references), and does not react with other potyviruses. In the USA, Agdia, Inc., (Elkhart, Indiana) will distribute the kit. The kit should be ordered directly from Agdia (219-264-2014, 800-622-4342), or (www.agdia.com), and is available in 1000 and 5000 test-well sizes.

3. **Read these guideline materials, and the materials from Agdia, carefully and store all the ELISA kit components at the recommended temperature to ensure their full shelf life.**

4. The Durviz kit was selected for the following reasons: a) this kit contains a universal monoclonal antibody specific for all plum pox virus isolates tested (about 84 isolates from *Prunus* including cherry, plum, apricot, nectarine, peach, and almond); b) this monoclonal antibody recognizes all plum pox serogroups (M, D, EA, and C); c) this monoclonal antibody reacts with plum pox isolates of unusual biology, molecular biology, and serology; d) this monoclonal antibody has a very low background for the duration of the test; and e) this monoclonal does not cross react (falsely identify) other potyviruses.

5. Each kit will include a plum pox virus positive control. The positive control will consist of the plum pox virus coat protein (PPV-CP) produced in a bacterium genetically modified with the PPV-CP gene. The coat protein gene is a part of the virus genetic material that produces a protein that covers and protects the virus in addition to other functions. The coat protein of any virus, or the CP gene, is incapable of causing infection. **No lyophilized (freeze-dried), infected plant material, live, infected plant material, or purified, infectious virus will be included in any kits used in the United States.**

Agdia will provide instructions with each kit that will describe the storage, and dilutions necessary for the positive control to give adequate absorbance readings at the end of the test.

6. **We suggest the use of a 100 ul volume in this procedure. Most ELISA microplate readers will accurately read ELISA plates with a reduced volume of 100 ul per well. If the manufacturer specifies that accurate readings can be taken at this volume, then use these test volumes throughout the test. If the manufacturer states that this smaller volume cannot be read accurately run all volumes at 200 ul and adjust all volumes in the protocol accordingly. You may want to contact the manufacturer for guidance if no minimum well volume is given for your unit. Volumes below the limits of your reader could result in inaccurate plate readings and survey data.**

B. Collection of Leaf Samples.

1. During the survey an effort should be made to collect sample leaves showing plum pox symptoms. Keep in mind that many infections may be without symptoms. Inspectors can familiarize themselves with typical plum pox leaf symptoms by visiting the plum pox virus web feature and plum pox image gallery at the American Phytopathological Society web site: www.apsnet.org, or the APHIS web site at www.aphis.usda.gov

2. As per the surveillance plan, four trees will constitute a **field sample**. One leaf will be collected from each scaffold branch of the tree (a scaffold branch forms the main framework of the tree). Therefore, a total of 12 or 16 leaves will be collected from the scaffold branches present in each of the four sample trees. The sampled leaves can be stored in one bag. **Each field sample will be divided and tested in a composite laboratory sample of no more than eight leaves. If a field sample consists of 12 leaves then the laboratory composite sample consists of 2, 6 leaf lab samples, and a field sample of 16 leaves will become 2, 8 leaf lab samples.**

3. Expanded leaves should be sampled from the middle third of this years' growth.

4. Plant material collected in the field should be placed in zip-lock plastic bags with the air expressed prior to sealing. Bags should be labeled using a smudge-proof permanent marking pen or pencil, or barcode labeled. The method of labeling of trees and sample bags will be determined by each testing location based on their experiences, equipment, and work force.

5. The leaf samples should be placed into the plastic bags without paper toweling, or any material that will keep the plant leaves wet. If leaves are excessively wet, the leaves should be shaken prior to bagging to remove water. Prolonged wetness promotes the growth of fungi and bacteria that contribute to a shortened storage life and degradation of samples that reduce absorbance readings.

6. Bags containing leaf samples should be kept cool at all times. Chilling promotes the extended storage life of samples by preventing wilting and decreasing microbial growth. Two alternative methods: 1) placing field samples into an ice chest, containing a suitable chilling agent, at the end of every collection row; or 2) temporarily storing field samples (with a frozen cold pack/refrigerant gel pack that is wrapped in a paper towel) in the collection container used in the field. When finished collecting in the block, samples should be transferred to an ice chest.

C. Storage of Leaf Samples.

1. At the conclusion of each collection period or day, samples should be transferred to a refrigerator or cold box and stored at 4°C in the testing laboratory, or other facility. **Leaf samples should not be frozen at any time prior to processing in ELISA.**

2. **Samples should be stored at 4°C for no more than 7 days prior to testing.** The detectable concentration of the virus will decrease with the aging of the leaves following sampling. Samples stored longer than 7 days will result in decreased absorbance values (the unit measured in ELISA).

3. **Samples that become partially decayed (a brown appearance, or moldy) should not be tested due to potentially decreased absorbance values. These materials must be recollected.**

D. Preparation of Tissue Samples for ELISA.

1. Remove the 6 or 8 composite leaves from each sample bag and neatly stack on top of each other in the same orientation. In other words, the leaf petioles are at one end of the stack, and the leaf tips at the opposite end of the stack:

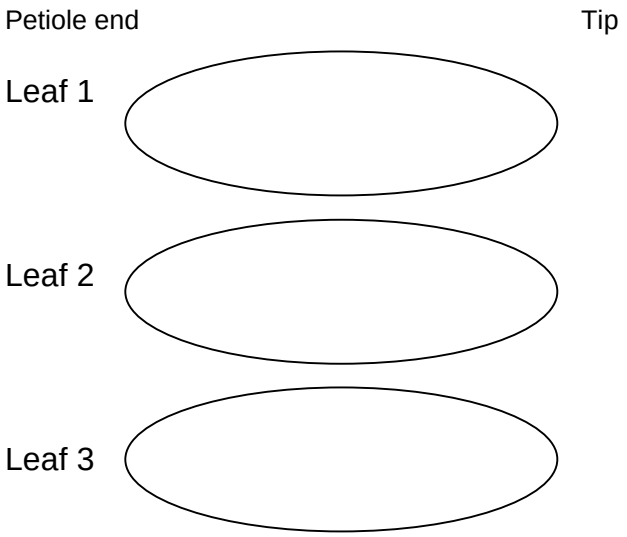

Figure 1. Diagram of stacked *Prunus* leaves for sampling.

2. From this stack hand tear or cut out the tissue for testing (if cutting, rinse the razor blade in water between each sample). Tear or cut the tissue from the petiole end of the leaf, **while excluding the petiole itself**. Weigh out 0.5 gm of tissue using an analytical balance. **Make sure that each leaf is represented in the 0.5 gm sample.**

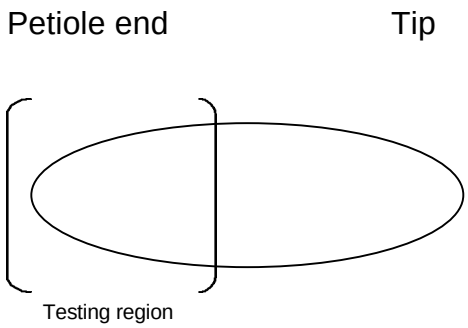

Figure 2. The test area of the leaf stack.

3. The samples can be stored 1-2 days after tissue is torn and weighed out if stored at 4°C (but not frozen) prior to extraction. If stored, the weighed samples should be held in a closed container or bag with a damp paper towel to prevent desiccation. Extraction buffer is added to the sample just prior to processing.

E. Preparing for the Test.

NOTE: All equipment (balances, pipettes, and readers) should be calibrated before testing begins to insure accuracy and reproducibility during the testing season. This will also prevent the needless wasting of reagents and incorrect dilutions due to inaccurate measuring devices. Good laboratory techniques should be followed at all times.

1. Make the necessary buffers as specified in the plum pox virus ELISA kit instructions including:

a) Carbonate antibody coating buffer (used in the antibody coating of ELISA plates), can be stored in the refrigerator for one week.
b) Phosphate buffered saline (PBS) (used as a component of the wash buffer and the extraction buffer) stored at room temperature for one week with sodium azide, without sodium azide should be made fresh.
c) Plate wash buffer (PBS-Tween) stored at room temperature for one week (if it contains sodium azide, otherwise make fresh)
d) Sample extraction buffer made fresh daily and kept cold on ice.
e) Diethanolamine substrate buffer stored in the refrigerator for one week.

See kit instructions and appendix for buffer recipes. Pre-made buffers (extraction buffer, PBS, and PBS-Tween wash buffer, etc.) and substrate will be available from Agdia and need to be made according to instructions by Agdia.
Sodium azide should be used with caution, please consult an MSDS sheet before handling this chemical.

2. PBS-based sample extraction buffer should be stored on ice during the day, and used ice cold.

3. Leaf samples can be ground using several devices: Use each as directed by the manufacturer.

-Mortars and pestles (cleaned between samples)
-Homex drill press from Bioreba with plastic extraction pouches (Agdia sells a modified device)
-Tissuemizer/Polytron/Tissue Tearor with sample tubes (cleaned between samples)
-Leaf roller press (cleaned between samples)

-Kleco tissue homogenization system and canisters (cleaned between samples).

4. Plates can be hand washed at a 45° angle with column twelve at the lowered end to prevent contamination from the positive control well. Specialized wash bottles with an 8 opening squirt head are available from Agdia. When hand washing, wash out the wells starting at column 12 then 11 then 10 and so on to avoid contaminating adjacent well rows. An automatic plate washer can also be used. For hand washing, the washes are generally 3 times for 5 minutes. However, the wash following sap (sample) incubation often requires 5 washes to remove *Prunus* sap that may remain in the microplate wells. A green hue may remain after the first wash that will require three or four additional washes. For automatic washing, please consult the instructions included with this apparatus, however please run a test to determine when the green hue from the sap is removed and adjust wash times accordingly. Adequate washing at this stage is critical.

5. The lot numbers for the kit components should be recorded for each plate in order to track any malfunctioning reagent. If mixed kits are used, each lot number should be recorded.

6. Helpful devices for testing include disposable reagent reservoirs/solution basins, plastic adhesive SealPlate™ film to seal plates and prevent spilling, and an Eppendorf repeat pipettor and combination tips (combi-tips) to rapidly pipet a repeated volume (such as the extraction buffer). All of the above devices are available from scientific supply vendors like LabSource, VWR, Fisher, etc.

F. Calculations of Dilutions:

The amount of buffer needed to fill a plate is calculated at 0.1 ml (100 ul) per well. There are 96 wells per plate so 9.6 mls are needed (0.1 ml X 96 = 9.6 ml). This figure is rounded to 10 ml per plate to provide sufficient buffer. This amount of buffer is then multiplied by the dilution factor to calculate the amount of antibody needed.

EXAMPLE FOR TEN PLATES:

10 plates X 10 ml = 100 ml.

For an antibody dilution of 1:100*:

100 ml of buffer X 1/100 dilution = 1 ml antibody in 100 ml of buffer.

For an antibody dilution of 1:1000*:

100 ml of buffer X 1/1000 dilution = 0.1 ml (100 ul) antibody in 100 ml of buffer.

* The above calculations are only examples. Check the kit directions for the appropriate dilutions. **Dilutions recommended by the manufacturer should be checked when new kits are used. If a pipette is not calibrated, you may come up short on the amount of solution available for dispensing. If this happens call a pipette calibration company in your area for service.**

Converting ml to ul:

To convert ml to ul, multiply by 1000.

1 ml = 1000 ul
0.1 ml = 100 ul
0.01 ml = 10 ul
0.001 ml = 1 ul

G. Running the PPV ELISA Test.

I. Antibody Coating.

1. Agdia will provide Microplates with the kits. The microplate is the Maxisorp™ plate manufactured by NUNC.

2. Microplates are prepared for ELISA by gently mixing the rabbit-PPV polyclonal antibody prior to dilution (provided in the kit usually as component A1-PPV) and coating with the appropriate antibody dilution *stated in the kit instructions* (usually 1:100). *Return the antibody stock bottle to the refrigerator immediately following dilution.* The coating antibody is prepared in carbonate coating buffer, pH 9.6 (see section H. II.), as per the kit instructions in an extremely clean and well-rinsed graduated cylinder. The antibody coating solution is poured into a reagent reservoir/basin and 100 ul loaded per well into ELISA plates using an 8 or 12 multi-channel pipette. The plates are sealed with adhesive covers and the plate is incubated in an oven pre-heated to 37°C for 4 hours, or incubated at 4°C for 15 hours (overnight). As an alternative to adhesive plate covers, plates can be stored in a humidity chamber by lining a plastic box with wet paper toweling and placing unsealed plates in the chamber/box which is covered and stored as above.

3. Antibody-coated plates are washed by dumping out the solution, and by inverting and shaking the plates in a downward motion over a sink or basin. The plates are washed three times, five minutes for each wash, with PBS-Tween

wash buffer as per section E.4 (for recipe see section H. II.). The washed plates are inverted (well side down) and tapped firmly on paper towels to remove excess wash buffer and empty the wells. The plates are ready for the addition of the sap sample **(do not store coated plates prior to loading)**.

II. Sample Tissue Extraction/processing.

1. Collected samples should be processed as soon as possible. Field samples can be stored at 4°C up to seven days prior to processing. Longer storage will decrease the amount of virus detected due to degradation of the virus in the aging plant tissue.

2. 0.5 gm of tissue (prepared as per section D) for processing should be ground with ice cold PPV extraction buffer at a ratio of 1:10 or 1:20 (one part tissue to10 or 20 parts buffer, for recipe see section H. II.). In other words, 0.5 grams of tissue ground in 5 mls or 10 mls of extraction buffer. Check the kit recommendations for appropriate sample dilutions. (The kit has recommended either 1:10 or 1:20. Sometimes plant tissues can decrease absorbance readings at dilutions of 1:10. One might split the difference with a 1:15 dilution of plant tissue to buffer. Because both have been listed it should be sufficient to use either ratio, however, you should consistently use one of the volumes to compare results over a season.)

-For samples processed with the leaf roller, feed the leaves through the roller and collect sap as rinsed off the roller with ice-cold extraction buffer. Rinse the rollers between each sample ground.

-For the drill press or Polytron/Tissuemizer/Tissue Tearor, samples can be prepared in bulk by adding the samples to bags or tubes, ice cold buffer added the day of extraction, and stored over ice until processed. Clean instrument between each use.

Any grinding implement that comes in direct contact with plant sap should be cleaned between each sample, usually by rinsing with copious amounts of distilled water.

3. Extracted sample sap should be stored in tubes on ice until loaded in ELISA plates. All processed samples should be loaded within 1-2 hours of grinding. **Ground samples should NOT BE stored overnight in the cold for loading the next day**. Storage of ground samples could lower the absorbance readings.

4. Make a loading diagram/plate map prior to loading samples and place in a page protector to prevent damage during loading. Each sample should be loaded into two adjacent wells.

5. For sample loading, a washed, antibody-coated, plate should be placed on a flat, frozen reusable refrigerant gel pack covered with a moist paper towel (to prevent slipping). The plate is positioned so that rows A-H are on the left side, and columns 1-12 are along the top. Starting in row A and column 1 and column 2, 100 ul of the sample should be loaded into each of two adjacent wells. The positive control, which will be provided in the kit and diluted and stored according to the manufacturers instructions, should be placed in wells G and H-12 to prevent possible contamination of test wells. A negative *Prunus* control, a positive PPV control, and a buffer control should be loaded in each plate. You should consistently add controls either first or last when loading a plate. Loaded plates should be kept cold while loading additional plates.

6. Any errors in loading should be noted immediately on the plate map/loading diagram before continuing.

7. Loaded plates should be sealed and stored at 4°C **for 16 hours** (not 12 hours) (overnight).

8. The next day wash plates about 4-5 times for 5 min each with PBS-Tween as stated in section E.4. This will remove *Prunus* sap that tends to stick to microplates following the first 1-2 washes. The plates are now ready for addition of the PPV-specific monoclonal antibody.

III. Adding the Specific Monoclonal Antibody.

1. The PPV-specific monoclonal antibody (usually kit component A2-PPV) is removed from the refrigerator, gently mixed, and then diluted as stated in the kit instructions (usually 1:1000) in an extremely clean and well-rinsed graduate cylinder. The monoclonal antibody dilution buffer is PBS plus 0.5% bovine serum albumin (BSA) (see section H. II.). *Return the antibody stock bottle to the refrigerator immediately following dilution.*

2. Pour into a reagent reservoir/basin and load 100 ul of solution to each well. Seal plates and incubate at 37°C for 2 hours.

3. After two hours, the wells are washed 3 times for 5 min each with PBS-Tween as per section E.4, and the inverted plates are tapped firmly on paper towels to remove excess liquid.

IV. Adding the Anti-mouse Alkaline Phosphatase-conjugated Antibody.

1. The conjugated antibody (usually kit component A3-PPV) is gently mixed and diluted 1:1000, or as instructed in the kit, in an extremely clean and well-rinsed graduate cylinder. *Return the antibody stock bottle to the refrigerator immediately following dilution.* The antibody is diluted in PBS solution. Pour into a reagent reservoir/basin and load 100 ul to each well, seal the plate, and incubate at 37°C for 2 hours.

2. After 2 hours, wash the plates 3 times for 5 min each with wash buffer (PBS-Tween) as described in section E.4.

V. Developing and Reading the Results.

1. During the 15-minute plate washing at the end of the anti-mouse conjugated antibody incubation (step IV), measure the appropriate substrate buffer (see section H.II.) and phosphate substrate (p-nitrophenylphosphate [PNP]). Prepare 1 mg/ml PNP in substrate buffer without touching the tablets (you may want to wear gloves to avoid contact with the skin) in an extremely clean and well-rinsed graduate cylinder, or disposable plastic tube. *Return the PNP bottle to the refrigerator immediately following addition.* Pour into the reagent reservoir/basin and load 100 ul to each well. Incubate at room temperature, covered from light (ie. cover with foil or in a covered dark box).

Note: Do not touch the PNP tablets or solution, or put fingers inside the reagent reservoirs. Phosphatases on fingers will utilize the PNP substrate and develop color before addition to the test plate. Do not expose to strong light. Light or contamination could cause background color in negative wells.

2. Read plates at 405 nm after incubation times of 15, 30, and 60 minutes, or as directed in the kit instructions. A positive reaction is recorded as 2.5 times the absorbance of the negative *Prunus* control (i.e. healthy *Prunus* tissue). If necessary, plates can be incubated for longer time. Plates that require several hours or longer (i.e. overnight) to develop the positive control wells could indicate a problem with a kit reagent, specifically the coating antibody. **Report this to Agdia, immediately.**

3. **Positive test results should be reported to the USDA-APHIS-PPQ Plum Pox Emergency Program Director, Gary Clement at 717-241-0705. Any sample testing positive should be listed as a suspect positive until laboratory confirmation by USDA-APHIS-PPQ in Beltsville, MD.** The sap of samples that test positive should be treated with a 10% bleach solution (a 10% solution of store-bought bleach) for 20 minutes. Remaining leaf tissue from samples that are suspected as positive for PPV should not be discarded.

Following notification of the PPV Emergency Program Director, arrangements will be made by the Program Director for shipment of suspect samples to APHIS-PPQ in Beltsville for testing. Following confirmatory testing, any PPV-positive leaf material must be destroyed by a minimum autoclave treatment of 30 minutes.

NOTE:
A. If a positive control well fails to record a positive reading, and no positive wells are recorded on the plate, the test is not valid and may have been handled incorrectly (i.e. missing reagent, faulty positive or antibody). The samples on that plate must be retested using newly ground leaf samples.

B. If a plate positive control fails to record a positive reading, yet a test well is positive, it indicates either a failure to add the positive control, or a problem with the positive control. If you experience a problem with the positive control, stop testing, and report it immediately to Agdia.

H. Appendix

I. Basic Equipment List:

Grinding mechanism:
- Homex-6 leaf drill press
- Tissuemizer, Polytron, Tissue Tearor
- Mortar and pestle
- Kleco homogenization canister system
- Leaf roller press

Analytical Balance

pH meter

Refrigerator or cold box for 4°C storage

ELISA plate reader with a 405 nm filter

Processing bags for the Bioreba Homex-6 unit

Incubator capable of 37°C

Stir plate

Bottles to store buffers

Plastic sample and plate storage boxes

500 and 1000 ml graduate cylinder
500 and 1000 ml flasks (for washing Polytron device, etc.)

1000 ul, 200 ul, 20 ul, and 10 ul pipettors and tips

8 or 12 channel multi-channel pipette and tips

Eppendorf repeat pipette with combination (combi) tips

Rinse bottle with eight well top for plate washing (Agdia)

Weigh boats

Small, disposable sample collection cups (for leaf roller press)

Small test tubes 5-10 ml volume and test tube racks (for the leaf roller press)

Large test tubes 50 ml volume and test tube racks (for Polytron device, etc.)

Sealplate™ Adhesive plate sealing film (to prevent spilling and evaporation)

Disposable reagent reservoirs/solution basins (non-sterile)

Wide opening pipette tips (the opening is large to accommodate viscous solutions like plant sap)

Distilled water

Ice

Reusable refrigerant ice blocks (gel or foam)

Liquid waste bin

Bleach

Parafilm

Aluminum foil

Razor blades

ELISA plate maps/loading diagrams

II. SOLUTIONS

GENERAL PROCEDURES FOR MAKING SOLUTIONS:

The following guidelines should be used when mixing solutions:

-A clean, well-rinsed, graduated cylinder is always used for measuring the buffer used in mixing antibody dilutions.

-A recently calibrated micropipet is used for measuring the antibody.

-Each Durviz antibody reagent is supplied diluted in 50% glycerol, and therefore must be gently mixed before measurement.

-All containers used for stored buffers should be emptied and washed and rinsed well with distilled water at the conclusion of each week.

-Low molarity HCl is used for decreasing the pH. Low molarity NaOH is used for increasing the pH.

-The instructions for the storage, dilution, and use of the protein positive control will be provided by Agdia and included with every kit.

BUFFERS

1. CARBONATE COATING BUFFER

1.59 grams, sodium carbonate (Na_2CO_3)
2.93 grams, sodium bicarbonate ($NaHCO_3$)
0.20 grams, sodium azide (NaN_3, optional)

Mix all chemicals to bring up to 800 ml and pH to 9.6, bring up to one liter with distilled water. Store at 4°C for one week.

2. PHOSPHATE BUFFERED SALINE (PBS) SOLUTION

Make fresh.

8.00 grams NaCl sodium chloride
0.20 grams KH_2PO_4 Potassium phosphate
1.15 grams Na_2HPO_4, sodium phosphate
0.20 grams KCl potassium chloride
0.20 grams NaN_3 sodium azide (optional)

Add the above chemicals to 800 ml of distilled water. Adjust pH to 7.2-7.4 and then bring solution up to 1 liter with distilled water.

3. PBS-Tween WASH BUFFER

PBS with Tween (PBST)

0.50 ml	Tween 20
500 ml	1X PBS
500 ml	Distilled water
0.20 grams	Sodium azide NaN_3 (optional, for preservation)

Store at 4°C for one week.

4. PPV EXTRACTION BUFFER

PBS, 2% PVP-10, 0.2% DIECA **Made fresh daily**

2.0 grams	DIECA (Diethyl dithiocarbamic acid, sodium salt)
20.0 grams	PVP-10, (Polyvinyl pyrrolidone)

A beaker with 800 ml of **PBS** should be moved to fume hood and the PVP added while in the hood. Allow this to stir for approximately 20 minutes to mix PVP into solution. Add DIECA and stir until all chemicals are in solution, approximately another 10 minutes. Adjust pH to 7.2-7.4, if needed, then bring solution to 1 liter **with PBS**.

5. SPECIFIC MONOCLONAL ANTIBODY DILUTION BUFFER

PBS plus 0.5% bovine serum albumin (BSA fraction V) **Made fresh daily**

5 grams of bovine serum albumin is added to 800 ml of PBS. When dissolved, bring up to 1 liter **with PBS**. Do not pH.

6. PNP SUBSTRATE BUFFER

To 800 ml distilled water:

Mix 97 ml	Diethanolamine.
0.1 grams	Magnesium chloride hexahydrate MgCl
0.2 grams	Sodium azide NaN_3 (optional, for preservation)

Adjust pH to 9.8 with concentrated hydrochloric acid (HCl). Bring solution up to 1000 ml with distilled water. Solution may be stored at 4°C in a foil covered or dark bottle, for later use that same week. Just before using, add p-nitrophenyl phosphate tablets (the substrate for the alkaline phosphatase) for a 1 mg/ml final concentration.

An alternative to making the above solution is to purchase the reagent and substrate buffer from Agdia or Sigma. Sigma carries PNP Fast-tabs in 5 mg and 20 mg concentrations. The 5 mg concentration makes 5 ml of a 1 mg/ml solution. The 20 mg concentration makes 20 ml of a 1 mg/ml solution. The fast tabs come with a substrate tablet and a buffer tablet that are added together in distilled water, dissolved at room temperature, and loaded in the plate. (The PNP Sigma Fast-tabs are often back-ordered)

NOTE: Do not contact the substrate solution with you hands or a stir bar that you, or others have touched due to phosphatases on your hands or objects that have been handled. Mix solution in an ultra clean and distilled water-rinsed bottle, 50 ml falcon disposable tubes or only with a clean glass stir rod in a well-cleaned beaker with no soap residue. If solution turns yellow prior to addition to plate, do not use.

Another alternative is to buy the Agdia, pre-made or pre-measured buffers and reagents. Go to www.agdia.com for other kit components listed in the section on the 2001 plum pox survey. There the kits and components are listed as well as the buffer packs and instructions for their use.

Instructions for the Agdia-supplied buffers:
(source is the Agdia website)

Agdia-supplied CARBONATE COATING BUFFER:

Prepare 1X working buffer directly from concentrate by diluting one volume of 10X carbonate coating solution with 9 volumes of distilled water.

Agdia-supplied PHOSPHATE BUFFERED SALINE (PBS):

Working 1X PBS buffer can be prepared directly from powder. Measure the distilled water into a container and add the buffer powder while stirring.

PBS buffer powder	9.55 grams
Distilled water	1000 mls

Optional: Adjust the pH of the 1X buffer, if necessary, until the pH is within the range of 7.2 to 7.4. The buffer should be very close to this pH at the making.

Agdia-supplied PPV SAMPLE EXTRACTION BUFFER:

Prepare a working 1X PPV extraction buffer. Measure PBS buffer into a container and add the DIECA and PVP-10 while stirring.

PBS buffer	900	ml
Sodium diethyldithiocarbamate (DIECA)	2.0	grams
Polyvinyl pyrrolidone (PVP-10)	20.0	grams

Adjust the pH of the 1X buffer until the pH is within the range of 7.2 to 7.4. **Adjust the final volume to 1000 ml with PBS buffer.**

Agdia-supplied PPV WASH SOLUTION:

1X washing solution can be prepared by diluting PBS buffer with distilled water and adding Tween-20.

Tween-20	0.50 ml
PBS buffer	500 ml
Distilled water	500 ml
Sodium azide NaN_3 (optional)	0.20 ml

(This wash buffer recipe using a 50% solution of PBS was recommended by Durviz, the kit supplier, however a standard PBS-tween wash solution also will work. This 50% recipe does have a benefit by reserving 50% of the PBS solution for use during the testing season. Use a wide mouth pipette tip for measuring the Tween-20).

Agdia-supplied SUBSTRATE BUFFER:

Prepare a 1X working PNP buffer solution directly from concentrate by diluting one volume of 5X PNP substrate solution with 4 volumes of distilled water. Check the concentration of the n-PNP tablets (usually each tablet is 5mg). If 5 mg tablets then this will make 5 mls of substrate solution at the required 1mg/ml concentration. Adjust the volumes and tablets for the number of plates to be developed.

References:

1. Candresse, T., Cambra, M., Dallot, S., Lanneau, M., Asensio, M., Gorris, M.T., Revers, F., Macquaire, A., Olmos, A., Boscia, D., Quiot, J.B., and J. Dunez. 1998. Comparison of monoclonal antibodies and polymerase chain reaction assays for the typing of isolates belonging to the D and M serotypes of plum pox potyvirus. Phytopathology 88(3), 198-204.

2. Myrta, A., Potere, O., Boscia, D., Candresse, T., Cambra, M., and V. Savino. 1998. Production of a monoclonal antibody specific for the El Amar strain of plum pox virus. Acta Virologica vol. 42, 248-250.

3. Myrta, A., DiTerlizzi, B., Boscia, D., Caglayan, K., Gavriel, I., Ghanem, G., Varveri, C., and V. Savino. 1998. Detection and serotyping of Mediterranean plum pox virus isolates by means of strain-specific monoclonal antibodies. Acta Virologica vol, 42 251-253.

4. Boscia, D., Zerandini, H., Cambra, M., Portere, O., Gorris, M.T., Myrta, A., DiTerlizzi, B., and V. Savino. 1997. Production and characterization of a monoclonal antibody specific to the M serotype of plum pox potyvirus. Eur. J. Plant Path. 103, 477-480.

5. Boscia, D., Cambra, M., Portere, O., Gorris, M.T., Myrta, A., DiTerlizzi, B., and V. Savino. 1996. Specific detection of the serotype M of plum pox potyvirus by means of monoclonal antibodies. Proceedings of the Middle European Meeting on Plum Pox '96, pp. 150-153.

6. Cambra, M., Asensio, M., Gorris, M.T., Perez, F., Camarasa, E., Garcia, J.A., Lopez-Moya, J.J., Lopez-Abella, D., Bela, C., and A. Sanz. 1995. Detection of plum pox virus using monoclonal antibodies to structural and non-structural proteins. Bulletin OEPP/EPPO Bulletin 24, 569-577.

***Acknowledgements*. I would like to thank everyone who provided additional thoughts, comments, and editorial advice during the construction of this document. I would like to specifically thank Gary Clement (USDA-APHIS), Vern Damsteegt (USDA-ARS), Renee DeVries (USDA-APHIS), Coanne O'Hern (USDA-APHIS), Anne Sindermann (Maryland Department of Agriculture), Arnold Tschanz (USDA-APHIS), Ruth Welliver (Pennsylvania Department of Agriculture), Marylou Polek CDFA, (Central California Tristeza Eradication Program), and Mike Tiffany (Agdia Inc.).**